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# Expression of Psychrophilic Genes in Mesophilic Hosts: Assessment of the Folding State of a Recombinant α-Amylase

GEORGES FELLER,* OLIVIER LE BUSSY, AND CHARLES GERDAY

*Laboratory of Biochemistry, Institute of Chemistry, University of Liège, B-4000 Liège-Sart Tilman, Belgium*

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**α-Amylase from the antarctic psychrophile *Alteromonas haloplanktis* is synthesized at 0 ± 2°C by the wild strain. This heat-labile α-amylase folds correctly when overexpressed in *Escherichia coli*, providing the culture temperature is sufficiently low to avoid irreversible denaturation. In the described expression system, a compromise between enzyme stability and *E. coli* growth rate is reached at 18°C.**

Psychrophilic enzymes possess specific properties, such as high activity at low temperatures and weak thermal stability, which promise to allow the use of these enzymes as industrial biocatalysts, as biotechnological tools, or for fundamental research (6, 8, 11). For instance, substantial energy savings can be obtained if heating is not required during large-scale processes which take advantage of the efficient catalytic capacity of cold-adapted enzymes in the range 0 to 20°C. The pronounced heat lability of psychrophilic enzymes also allows their selective inactivation in a complex mixture, as illustrated by an antarctic bacterial alkaline phosphatase which is available for molecular biology research (7). Finally, psychrophilic enzymes represent the lower natural limit of protein stability (3) and are useful tools for studies in the field of protein folding.

Large-scale fermentation of psychrophilic microorganisms suffers from two main drawbacks, however: the low production levels of wild strains and the prohibitive cost of growing wild strains at low temperatures. A possible alternative is to overexpress the gene coding for a psychrophilic protein in a mesophilic host for which efficient expression systems have been designed. In this context, two crucial questions remain to be solved: (i) what is the folding state of an enzyme normally synthesized at 0°C when it is expressed by the mesophilic genetic machinery at higher temperatures, and (ii) is there a temperature at which a compromise can be reached between the stability of the psychrophilic enzyme and the mesophilic growth rate? To address these questions, the heat-labile α-amylase from the antarctic psychrophile *Alteromonas haloplanktis* (2, 4) was expressed in *Escherichia coli* at various temperatures.

**Construction of the expression vector and α-amylase production.** The α-amylase gene (2) was cloned downstream from the *lacZ* promoter in pUC12 by ligating the *Sma*I site of the polylinker to the *Hpa*I site located 60 nucleotides upstream from the formylmethionine codon. This construction is devoid of the C-terminal peptide cleaved by the wild strain following α-amylase secretion. The recombinant enzyme was expressed in *E. coli* RR1 with the constitutive assistance of *lacZ* (without IPTG [isopropyl-β-D-thiogalactopyranoside] induction) in a medium containing 16 g of bactotryptone, 16 g of yeast extract, 5 g of NaCl, 2.5 g of $K_2HPO_4$, 0.1 μM $CaCl_2$, and 100 mg of ampicillin per liter. The effect of the culture temperature on α-amylase production by *E. coli* is illustrated in Fig. 1. Within the range of temperatures used, maximal enzyme production was reached below 18°C, whereas higher temperatures induced a gradual decrease of α-amylase activity in cultures. Three independent cultures were pooled for the purification of the recombinant enzymes produced at 18 and 25°C.

**α-Amylase purification.** The gram-negative *A. haloplanktis* was cultivated at 4°C, and α-amylase was purified from the culture supernatants by ion-exchange chromatography on DEAE-agarose followed by gel filtration on Sephadex G-100 and Ultrogel AcA54 as previously described (2, 4). The recombinant α-amylases were purified by the protocol developed for the wild-type enzyme except that concentration by ammonium sulfate precipitation at 70% saturation was required before the first chromatographic step. Recombinant enzyme production at 18 and 25°C ranged between 60 and 100 mg/liter of culture, which corresponds to a 10-fold improvement over production by the wild strain.

**Characterization of the recombinant α-amylases.** N- and C-terminal amino acid sequences (determined on an Applied Biosystems Procise analyzer and by carboxypeptidase Y digestion, respectively) of α-amylase produced at 18 and 25°C indicated that the signal peptide is correctly cleaved in *E. coli* and that no additional posttranslational cleavage occurred. The isoelectric point (5.5) and the molecular mass (49,340 Da as determined from the sequence and 49,342 ± 8 Da as determined from electrospray mass spectroscopy measurements) were identical to the values recorded for the wild-type enzyme. Dynamic light scattering (DynaPro-801; DLS Instruments) also showed that the purified recombinant enzymes are homogeneous, without any evidence of aggregated forms.

**Comparison of the wild-type and recombinant α-amylases.** Several properties of the wild-type enzyme produced at 4°C and the recombinant α-amylase expressed in *E. coli* at 18°C were compared (Table 1).

**(i) Kinetic and ion binding parameters.** 4-Nitrophenyl-α-D-maltoheptaoside-4,6-O-ethylidene (EPS) was used as the substrate in a coupled assay with α-glucosidase at 25°C. The absorption coefficient for 4-nitrophenol was 8,990 $M^{-1} \cdot cm^{-1}$ at 405 nm, and a stoichiometric factor of 1.25 was applied for $k_{cat}$ (turnover number) calculation. Dissociation constants were determined by activation kinetics following $Cl^-$ or $Ca^{2+}$ titration of the apoenzyme obtained by dialysis against 25 mM HEPES-NaOH (pH 7.2) and 25 mM HEPES-NaOH–5 mM EGTA (pH 8.0), respectively. The saturation curves were computer fitted by a nonlinear regression analysis of the Hill equation in the form $v = k_{cat}$

* Corresponding author. Mailing address: Laboratory of Biochemistry, Institute of Chemistry B6, University of Liège, B-4000 Liège-Sart Tilman, Belgium. Phone: 32-4-366 33 43. Fax: 32-4-366 33 64. E-mail: gfeller@ulg.ac.be.

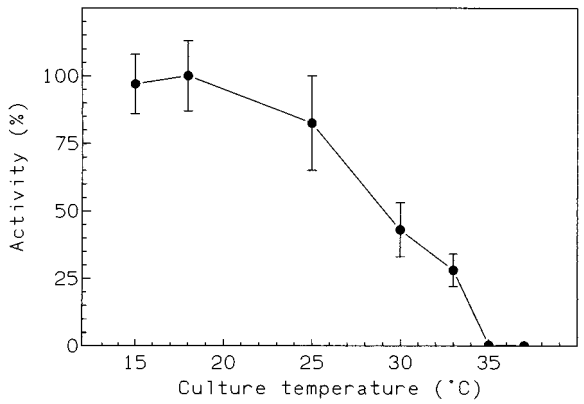


FIG. 1. Temperature dependence of α-amylase production by *E. coli*. Results are expressed as percent mean maximal activity recorded at 18°C.

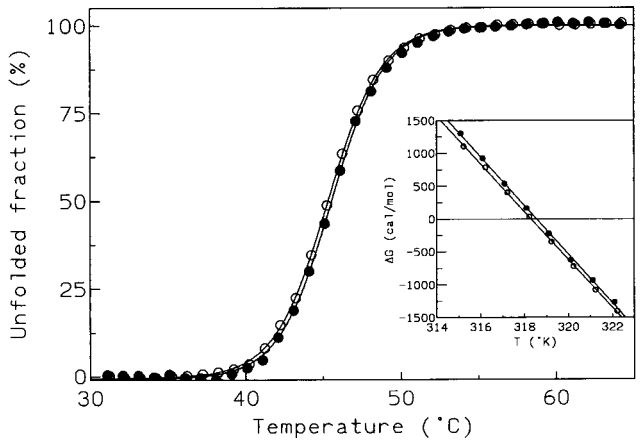


FIG. 2. Heat-induced unfolding transitions of the wild-type α-amylase (●) and the recombinant enzyme produced at 18°C (○). The fraction of protein in the unfolded state ($f_U$) was calculated as follows: $f_U = (y_F - y)/(y_F - y_U)$, where $y_F$ and $y_U$ are the fluorescence intensities of the native and the fully unfolded states, respectively, and $y$ is the fluorescence intensity at a given temperature. The inset shows a plot of $\Delta G$ as a function of the temperature around the melting point ($T_m$), where $\Delta G = 0$.

$[I]^h/K_d + [I]^h$ where $[I]$ is the ion concentration and $h$ is the Hill coefficient. The free calcium concentrations were set by calcium titration in the presence of 5 mM EGTA at pH 8.0. Kinetic parameters ($k_{cat}$, $K_m$ and $k_{cat}/K_m$) for the hydrolysis of EPS as well as dissociation constants ($K_d$) for calcium and chloride ions were found to be identical in the wild-type and recombinant enzymes produced at 18°C (Table 1). Owing to the stringent structural requirements for functional active site and ion binding site conformation, it can be safely concluded that the recombinant enzyme is properly folded at 18°C.

**(ii) Disulfide bond integrity.** Free thiol content was determined by DTNB (5,5′-dithiobis-2-nitrobenzoic acid) titration of the unfolded enzyme in 8 M urea in order to promote −SH group accessibility. The eight cysteine residues of *A. haloplanktis* α-amylase are engaged in disulfide linkages (4). Thus, the lack of free sulfhydryl groups, as detected by DTNB titration of both the native and the unfolded enzymes (Table 1), indicates that the four disulfide bonds are formed in the recombinant α-amylase samples.

**(iii) Conformational stability.** Fluorescence intensity of α-amylases (50 μg/ml) was recorded in 30 mM MOPS (morpholinepropanesulfonic acid)–50 mM NaCl–1 mM $CaCl_2$ (pH 7.2) at a scanning rate of 1°C/min and at an excitation wavelength of 280 nm and an emission wavelength of 347 nm with a Perkin-Elmer LS 50 spectrofluorimeter. Raw data were corrected for thermal dependence of the fluorescence by using the slopes of the pre- and posttransition regions as described elsewhere (10). The conformational stability ($\Delta G_{N \Leftrightarrow U}$) was determined by reversible, thermally induced unfolding recorded by fluorescence. Both the wild-type and the recombinant α-amylases have melting point ($T_m$) values of 45 ± 0.2°C and display the same cooperative transition (Fig. 2). Consequently, plots of $\Delta G$ as a function of $T$ (constructed by using the relation $\Delta G = -RT\ln K$, where $K$ = fraction unfolded/fraction folded) are similar (Fig. 2, inset). These results indicate that the weak interactions stabilizing the folded state of the wild-type and recombinant α-amylases are quantitatively identical.

**Expression at 25 and 37°C.** When cultures of the recombinant *E. coli* are carried out at 25°C, all parameters determined by activation kinetics and independent of the enzyme concentration, such as $K_m$ and $K_d$, remain constant, as does the free sulfhydryl content (Table 1). This indicates that the native enzyme fraction is correctly folded. By contrast, the $k_{cat}$ of the recombinant α-amylase is reduced by about 20%, suggesting the occurrence of a corresponding inactive fraction. When expressed at 37°C, no α-amylase activity is recorded; the recombinant heat-labile enzyme could fail to fold at this high temperature, or its denaturation rate could exceed its synthesis rate. Furthermore, Western blotting with rabbit polyclonal antibodies to α-amylase detects only trace amounts of the recombinant gene product, suggesting that the denatured enzyme is quickly degraded by the *E. coli* cell.

**Conclusions.** We have previously shown that cloning of a psychrophilic gene in *E. coli* and detection of the gene product can be achieved by careful control of the culture conditions: overnight incubation at 25°C of transformed cells followed by 1 to 2 days of incubation at 4°C produced halos of substrate hydrolysis on agar plates (5). The folding state of the recombinant psychrophilic enzymes (e.g., fully or partly active, native or marginal stability, etc.), however, was unknown. The results presented here demonstrate that the genuine properties of a psychrophilic enzyme are preserved when it is expressed in a

TABLE 1. Kinetic parameters, dissociation constants, and free thiol groups for the wild-type and recombinant α-amylases

| α-Amylase | $k_{cat}$ ($s^{-1}$) | $K_m$ (μM) | $k_{cat}/K_m$ ($s^{-1} \cdot \mu M^{-1}$) | $K_d$ | | Cysteines[a] ($mol^{-1}$) | Free thiol ($mol^{-1}$) |
|---|---|---|---|---|---|---|---|
| | | | | $Cl^-$ (mM) | Ca (M) | | |
| Wild-type (produced at 4°C) | 780 ± 25 | 174 ± 8 | 4.6 | 5.9 ± 0.2 | $2.10^{-8}$ | 8 | 0.03 |
| Recombinant (produced at 18°C) | 792 ± 34 | 168 ± 14 | 4.7 | 6.1 ± 0.2 | $2.10^{-8}$ | 8 | 0.05 |
| Recombinant (produced at 25°C) | 609 ± 29 | 186 ± 22 | 3.3 | 6.0 ± 0.3 | $2.10^{-8}$ | 8 | 0.05 |

[a] From the amino acid sequence.

mesophilic host, providing the culture temperature is sufficiently low to allow correct folding and to avoid irreversible denaturation. In our expression system, a compromise is reached between the stability of the psychrophilic enzyme and the growth rate of the mesophilic host by cultivating the recombinant *E. coli* at 18°C. It should be noted that commonly used *E. coli* strains have different growing capacities at that temperature. We found *E. coli* RR1, HB101, or XL1-Blue (Stratagene) suitable for these culture conditions (the generation times are about 3 h, and stationary phase is reached after approximately 30 h), whereas *E. coli* DH5α grows twice as slowly at 18°C.

The lack of α-amylase expression at 37°C is not an isolated case: under the same conditions, lipases and proteases (1, 5, 9) from antarctic psychrophiles were not expressed in an active form. This illustrates the general heat lability of psychrophilic enzymes, which is thought to arise from their flexible conformation, allowing high catalytic activity at temperatures close to 0°C (3).

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